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Immunogenic properties of amyloid beta oligomers

Indre Dalgediene^{1*}, Rita Lasickiene¹, Rima Budvytyte², Gintaras Valincius², Ramune Morkuniene³, Vilmante Borutaite³ and Aurelija Zvirbliene¹

Abstract

Background: The central molecule in the pathogenesis of Alzheimer's disease (AD) is believed to be a small-sized polypeptide – beta amyloid (A β) which has an ability to assemble spontaneously into oligomers. Various studies concerning therapeutic and prophylactic approaches for AD are based on the immunotherapy using antibodies against A β . It has been suggested that either active immunization with A β or passive immunization with anti-A β antibodies might help to prevent or reduce the symptoms of the disease. However, knowledge on the mechanisms of A β -induced immune response is rather limited. Previous research on A β 1-42 oligomers in rat brain cultures showed that the neurotoxicity of these oligomers considerably depends on their size. In the current study, we evaluated the dependence of immunogenicity of A β 1-42 oligomers on the size of oligomeric particles and identified the immunodominant epitopes of the oligomers.

Results: Mice were immunized with various A β 1-42 oligomers. The analysis of serum antibodies revealed that small A β 1-42 oligomers (1–2 nm in size) are highly immunogenic. They induced predominantly IgG2b and IgG2a responses. In contrast, larger A β 1-42 oligomers and monomers induced weaker IgG response in immunized mice. The monoclonal antibody against 1–2 nm A β 1-42 oligomers was generated and used for antigenic characterization of A β 1-42 oligomers. Epitope mapping of both monoclonal and polyclonal antibodies demonstrated that the main immunodominant region of the 1–2 nm A β 1-42 oligomers is located at the amino-terminus (N-terminus) of the peptide, between amino acids 1 and 19.

Conclusions: Small A β 1-42 oligomers of size 1–2 nm induce the strongest immune response in mice. The N-terminus of A β 1-42 oligomers represents an immunodominant region which indicates its surface localization and accessibility to the B cells. The results of the current study may be important for further development of A β -based vaccination and immunotherapy strategies.

Keywords: Alzheimer's disease (AD), Amyloid beta (A β), Neurotoxicity, Immunogenicity, Neurotoxic oligomers, Epitope mapping

Background

Alzheimer's disease (AD) is the most common progressive neurodegenerative disease in elderly people. The disease manifests as decline in cognitive functions and memory loss followed by changes in personality and mental state. It is known that while the disease evolves the main changes take place in parts of the brain responsible for the cognitive functions and memory. One of the central molecules involved in the pathogenesis of AD is

thought to be a small sized polypeptide – beta amyloid (A β) which has an ability to assemble spontaneously into oligomers [1]. It has been shown that these oligomers depending on their size and shape have different neurotoxic effects on rat brain cell cultures [2]. Several therapeutic and prophylactic approaches based on the anti-amyloid immunotherapy have been suggested for AD and clinical trials have been started with either active immunization with A β protein or passive immunization with anti-A β antibodies. However, acute brain tissue inflammation while using these treatments has been reported and several clinical trials were cancelled due to this side effect [3-5]. Therefore, better

* Correspondence: indre.dalgediene@bti.vu.lt

¹Institute of Biotechnology, Vilnius University, V. Graiciuno str. 8, LT-02241, Vilnius, Lithuania

Full list of author information is available at the end of the article

understanding of the antigenic properties of A β and its effects on the functions of immune cells may lead to the improvement of the anti-amyloid immunotherapy.

In the current study we investigated the immunological and antigenic properties of A β 1-42 oligomers. We demonstrated that various A β 1-42 oligomers differ in their capacity to induce antibody response in experimental mice. We also employed the monoclonal and polyclonal antibodies generated against A β 1-42 oligomers to identify their immunodominant epitopes.

Methods

A β 1-42 oligomers

All A β 1-42 oligomers used in this study were prepared and characterized as described previously [2,6,7]. Synthetic A β 1-42 peptide was purchased from American Peptide Company (Sunnyvale, California, USA). Millipore purified water (18.2 M Ω cm) was used throughout the work.

Briefly, broad size range (1–15 nm in size) A β 1-42 oligomers were prepared by dissolving 0.3 mg of A β 1-42 and incubating it in 130 μ l hexafluoroisopropanol (HFIP) for 20 min in 1.5 ml vials, followed by addition of 900 μ l H₂O and 20 min incubation in the resulting HFIP/water mixture. Subsequently, the samples were centrifuged at 12,000 g for 15 min. The solvent was evaporated from resulting supernatant under nitrogen stream for 5 min. Then the samples were incubated in closed vials for 24 h with constant stirring at room temperature.

The protocol described above was modified for producing smaller A β 1-42 oligomeric particles. 1 mg of peptide was dissolved in 400 μ l HFIP and incubated for 30–60 min at room temperature. 100 μ l of the resulting seedless solution was added to 900 μ l H₂O in a siliconized vial. After 10–20 min of incubation at room temperature the samples were centrifuged for 15 min at 12,000 g, the supernatant was transferred to a new siliconized tube and HFIP was evaporated in ambient air for 1 h. Then the samples were incubated in closed vials for 24 h in a thermostatic bath, at +20°C. The fraction of A β 1-42 oligomeric particles of 1–2 nm size was produced by this protocol.

To generate larger, typically 5–10 nm A β 1-42 oligomers, after the centrifugation the supernatant was transferred to a nonsiliconized vial and gently purged with nitrogen for 7 min. Then the preparation was stirred in the same vial at ~500 rpm for 24 h using a magnetic Teflon-coated stirring bar.

Finally, the A β 1-42 monomers were formed by dissolving A β 1-42 peptide in Millipore purified water immediately before the experiments. All solutions of prepared A β 1-42 oligomers were stored at –20°C no longer than for 24 h.

Shortly, to assess the size and morphology of the preparations of A β 1-42 oligomers, atomic force microscopy (AFM; Agilent 5500, Santa Clara, California, USA) was used in the tapping mode. According to the manufacturer's recommendations, the probe tip diameters were between 16 and 20 nm. About 20 μ l of a 10 μ M A β 1-42 solution was spotted on freshly cleaved mica (SPI Supplies, West Chester, Pennsylvania, USA), incubated at room temperature for 10 min and rinsed with Millipore purified water, then blown dry with a nitrogen stream. Images were acquired at scan rates between 0.5 and 1 Hz with the drive amplitude and force kept to a minimum. The particle size was estimated by measuring the profile of the sample within the sample plane.

Recombinant antigens

Recombinant proteins used for the analysis of antibody specificity by ELISA: hamster polyomavirus major capsid protein VP1 (VP1) and human metapneumovirus (hMPV) nucleocapsid (N) protein (hMPV N) were described previously [8,9].

Recombinant A β 1-40 protein fused with thioredoxin (Trx-A β 1-40) and expressed in *E. coli* was used for the analysis of antibody reactivity by Western blot. Shortly, thioredoxin gene was fused with A β 1-40 gene at its N-terminus and cloned into expression vector pET3a. Fused protein Trx-A β 1-40 was expressed in *E. coli* strain DH5 α and purified under denaturing conditions using Ni chelating column.

Immunization of mice and generation of monoclonal and polyclonal antibodies

BALB/c mice were bred and maintained in an animal facility at the Department of Immunology of the Centre for Innovative Medicine (Vilnius, Lithuania). The groups of 4 female mice aged 6–8 weeks per each antigen were immunized with A β 1-42 broad size range oligomers, 1–2 nm A β 1-42 oligomers, 5–10 nm A β 1-42 oligomers and A β 1-42 monomers (non-treated peptide). Control group of BALB/c mice (n = 4) received PBS injections. All injections were subcutaneous. The dose was 50 μ g of oligomers or peptide per mouse. For the primary immunizations the antigens were emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, Missouri, USA). The second immunization followed on day 28 with the antigens dissolved in PBS. Antiserum samples were collected on day 14 after the first and second immunizations and tested by an indirect enzyme-linked immunosorbent assay (ELISA) for the presence of IgG antibodies specific to A β 1-42 oligomers and the monomers. The spleen cells of the mouse with the highest antibody titre were used for the generation of hybridomas [10]. Three days after the boost

immunization the spleen cells of the mouse were fused with Sp2/0-Ag14 mouse myeloma cells using polyethylene glycol 1500 (PEG/DMSO solution, HybriMax, Sigma-Aldrich). Hybrid cells were selected in growth medium supplemented with hypoxanthine, aminopterin and thymidine (50× HAT media supplement, Sigma-Aldrich). Samples of supernatant from wells with viable clones were screened by an indirect ELISA. Hybridomas secreting A β 1-42 specific antibodies were subcloned twice by a limiting dilution method. Hybridoma cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) containing 15% fetal calf serum (Biochrom) and antibiotics. Antibodies were isotyped using Monoclonal Antibody Isotyping Kit I (HRP/ABTS) (Pierce Biotechnology, Rockford, Illinois, USA) in accordance with the manufacturer's protocol. All procedures involving experimental mice were performed under controlled laboratory conditions in strict accordance with the Lithuanian and European legislation.

Indirect enzyme-linked immunosorbent assay (ELISA) analysis for anti-A β 1-42 antibodies

Microtiter plates (Nunc MaxiSorp, Nunc, Roskilde, Denmark) were coated with 100 μ l/well of either A β 1-42 oligomers or A β 1-42 peptide dissolved in the coating buffer (0.05 M sodium carbonate, pH 9.5) to a concentration of 5 μ g/ml. For the coating with A β 1-42 oligomers the plates were incubated overnight at +4°C. The A β 1-42 peptide was dried in the plates by incubating overnight at +37°C. The coated plates were blocked with 250 μ l/well of PBS with 2% BSA for 1 h at room temperature (RT). Then plates were rinsed twice with PBST (PBS with 0.1% Tween-20). Antiserum samples, hybridoma growth medium or polyclonal antibodies were diluted in PBST, added to the wells (100 μ l/well) and incubated for 1 h at RT. The plates were then incubated for 1 h with Goat Anti-Mouse IgG (H+L)-HRP Conjugate (Bio-Rad, Hercules, California, USA) diluted 1:5000 in PBST. The enzymatic reaction was visualized by the addition of 100 μ l of "NeA-Blue" TMB solution (Clinical Science Products, Mansfield, Massachusetts, USA) to each well. The reaction was stopped by adding 50 μ l/well of 10% sulphuric acid solution. The optical density (OD) was measured at 450 nm (reference filter 620 nm) in a microplate reader (Sunrise Tecan, Männedorf, Switzerland).

SDS-PAGE and western blot analysis

The samples of recombinant fused protein Trx-A β 1-40, *E. coli* DH5 α lysate and HeLa lysate were boiled in a reducing sample buffer and separated in 15% polyacrylamide gel electrophoresis (PAGE) in SDS-Tris-glycine buffer. Proteins were visualized by staining with Coomassie Brilliant blue (Sigma-Aldrich). The proteins from the unstained SDS-PAGE gel were blotted onto Roti[®]-PVDF

membrane (Carl Roth, Karlsruhe, Germany) by semidry electro-transfer. The membrane was blocked with 2% milk powder in PBS for 2 h at RT and rinsed with PBST. The membrane was then incubated for 1 h at RT with primary antibodies at working dilution in PBST with 2% milk powder and subsequently incubated with Goat Anti-Mouse IgG (H+L)-HRP Conjugate (Bio-Rad) diluted 1:4000 in PBST with 2% milk powder. The enzymatic reaction was developed using 4-chloro-1-naphthol and H₂O₂ (Fluka, Milwaukee, Wisconsin, USA). For the analysis of antisera, they were diluted 1:1000 in PBST. For the analysis of monoclonal antibodies, undiluted hybridoma supernatant was used.

Characterization of A β 1-42 epitopes recognized by polyclonal and monoclonal antibodies

For the epitope mapping, the collection of synthetic, overlapping, A β 1-42 spanning, 13 amino acids (aa) in length and linked with biotin-SGSG at the N-terminus peptides was used (Metabion, Martinsried, Germany). Microtiter plates (Nunc MaxiSorp, Nunc) were coated with streptavidin dissolved in deionized H₂O (2 μ g/ml) by incubating overnight at +37°C. The coated plates were blocked with 2% BSA in PBS. Synthetic peptides with biotin were added to the wells diluted in PBST at concentrations recommended by the manufacturer and incubated for 1 h at RT. Hybridoma supernatant (undiluted) and polyclonal antibodies (diluted 1:1000 in PBST) were added to the wells (100 μ l/well) and incubated for 1 h at RT. The plates were incubated for 1 h with Goat Anti-Mouse IgG (H+L)-HRP Conjugate (Bio-Rad) diluted 1:5000 in PBST. The enzymatic reaction was visualized with TMB as described above.

Results

Immune response to A β 1-42 oligomers in mice

To investigate the immunogenicity of various A β 1-42 oligomer forms, four groups of BALB/c mice were exploited. Each group of mice was injected with the same doses (50 μ g per mouse) of the antigens: broad size range A β 1-42 oligomers, A β 1-42 oligomers with particle size of 1–2 nm, A β 1-42 oligomers with 5–10 nm particle size and A β 1-42 monomers. The levels of antigen-specific IgG antibodies in the antiserum samples were estimated by calculating the antibody titres in ELISA against the antigens used for the immunization. After the first immunization, the titres of antigen-specific IgG antibodies in all groups of immunized mice did not exceed 1:100 (data not shown). Although the adjuvant was used for the primary immunization to enhance the immune response, the adjuvant might have also disrupted the oligomeric structure of the antigens. After the secondary immunization applied without an adjuvant there was a significant difference in antibody

titres between all groups of immunized mice (Figure 1). The highest antibody response was observed in mice immunized with 1–2 nm A β 1-42 oligomers. The titres of antigen-specific IgG were almost 3 times higher in this group of mice as compared to that immunized with broad size range oligomers (1:2000 and 1:700, respectively). In contrast, the titres of antigen-specific IgG in the group of mice immunized with 5–10 nm A β 1-42 oligomers did not exceed 1:500. Thus, the series of the strongest A β 1-42 immunogens varied from 1–2 nm oligomers to the broad size range oligomers and 5–10 nm oligomers. As expected, A β 1-42 monomers demonstrated the weakest immunogenic properties giving antibody titres about 1:200 (Figure 1). Moreover, antisera raised against 1–2 nm A β 1-42 oligomers showed in ELISA 4 times lower titres with A β 1-42 monomers as compared to 1–2 nm A β 1-42 oligomers: 1:500 and 1:2000, respectively. Antisera collected from the control group of mice injected with PBS alone showed only background level activity with antibody titres less than 1:20. These results demonstrated that A β 1-42 oligomeric structure is important in the activation of B cells and induction of the antibody response. To evaluate the involvement of helper T cells in B cell activation, the subclass distribution of antigen-specific IgG antibodies in the pool of antisera of mice immunized with 1–2 nm A β 1-42 oligomers was analysed. The majority of the IgG antibodies (57%) raised against 1–2 nm A β 1-42 oligomers were of IgG2a isotype (Table 1). In contrast, antigen-

specific antibodies of IgG1 and IgG3 subtypes were presented to a less extent (19% and 2% of total IgG, respectively).

Generation of monoclonal antibodies against A β 1-42 oligomers

Spleen cells of mice with the highest antibody titre induced by 1–2 nm A β 1-42 oligomers were used to generate the monoclonal antibodies (MAbs). One stable hybridoma clone 11E12 producing A β 1-42-specific MAb of IgG2b subtype was generated. An indirect ELISA with different A β 1-42 oligomers and the monomers revealed a strong reactivity of the MAb 11E12 with 1–2 nm A β 1-42 oligomers: titres of antigen-specific antibodies in the hybridoma supernatant were 1:2000. In contrast, the reactivity of the MAb 11E12 with 5–10 nm oligomers and the monomeric peptide was significantly lower: titres in ELISA were 1:80 and 1:50, respectively (data not shown). The specificity of the MAb to the A β 1-42 sequence was also confirmed by Western blot analysis of recombinant fused protein Trx-A β 1-40. The MAb 11E12 reacted specifically with recombinant Trx-A β 1-40 but did not react with *E. coli* DH5 α lysate and HeLa cell lysate used as negative controls (Figure 2A). Antiserum of the mouse immunized with 1–2 nm A β 1-42 oligomers and used for the generation of monoclonal antibody 11E12 also showed reactivity with recombinant fused protein Trx-A β 1-40 (Figure 2B). In contrast, pools of antisera raised against broad size range A β 1-42 oligomers, 5–10 nm

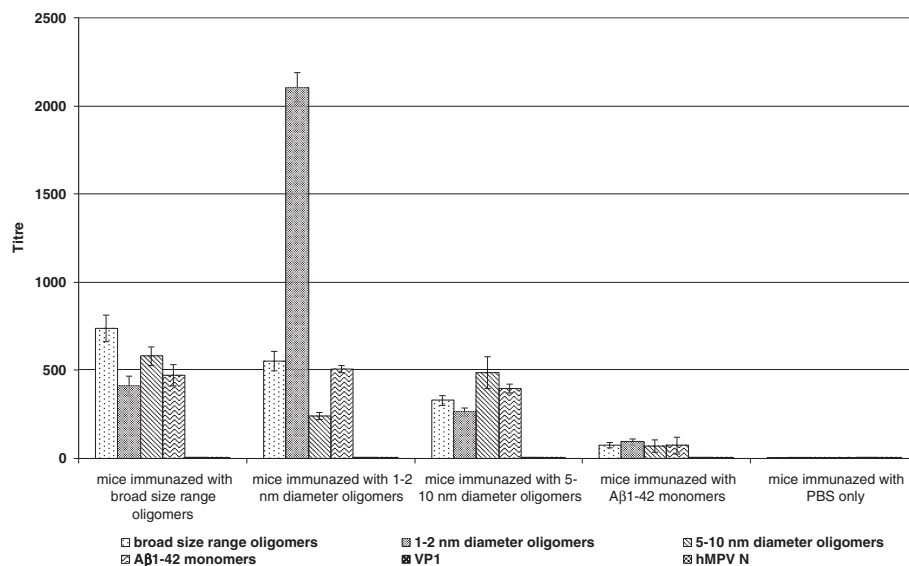


Figure 1 Titres of A β 1-42 specific IgG in the antisera of immunized mice. The immunogenicity of A β 1-42 oligomers was determined by an indirect ELISA using mouse antisera raised against A β 1-42 oligomers. The microtiter plate was coated with various A β 1-42 oligomers, A β 1-42 peptide (monomers) or irrelevant antigens and subsequently incubated with the antisera collected on the 14th day after the second immunization.

Table 1 Distribution of the subtypes of IgG antibodies raised against 1–2 nm A β 1-42 oligomers, expressed in percents of total A β 1-42 specific IgG

Subtype	Percents of total A β 1-42 specific IgG, %
IgG1	19
IgG2a	57
IgG2b	22
IgG3	2

A β 1-42 oligomers and A β 1-42 monomers showed week or no reactivity with recombinant fused protein Trx-A β 1-40 (data not shown).

Mapping of A β 1-42 epitopes recognized by polyclonal and monoclonal antibodies

To investigate the antigenic structure of A β 1-42 oligomers, we have employed a series of overlapping 13 aa-long synthetic peptides spanning the A β 1-42 sequence (Table 2). The MAb 11E12 reacted exclusively with the peptide representing the N-terminal sequence (aa 1–13, Table 2). Antiserum of the mouse immunized with 1–2 nm A β 1-42 oligomers and used for the generation of monoclonal antibody 11E12 showed strong reactivity exclusively with peptides #1 and #2 representing the N-terminus of A β 1-42 protein (aa 1–19). Pools of antisera raised against the broad size range A β 1-42

oligomers and 5–10 nm A β 1-42 oligomers showed a week positive reaction also with the N-terminal peptide #1 and did not show any reactivity with other peptides. Thus, regardless of the form of A β 1-42 oligomers the N-terminus was identified as an immunodominant region of A β 1-42 oligomers.

Discussion

The attempts to use active immunization with A β or passive immunization with anti-A β antibodies represent promising new strategies in the immunotherapy of AD. However, the results of previous preclinical and clinical studies of AD immunotherapy were controversial [3,5]. In some cases acute brain inflammation occurred [3]. A number of questions on the origin and mechanism(s) of these unwanted side effects still remains unanswered. It is not yet known what impact on the pattern of the immune response various aggregate forms of A β may have. To elucidate the mechanisms of anti-A β immune response, naturally occurring antibodies against A β have been investigated [11]. It is also possible to focus on various artificial A β aggregate forms and gain information on their immunogenic and antigenic properties using laboratory animal models.

In the current study, we have investigated the immunogenicity of various A β 1-42 oligomers including the small-sized oligomers that were shown to be highly neurotoxic *in vitro* [2,12]. The analysis of the humoral immune response in mice immunized with different A β 1-42 oligomers revealed that their immunogenicity strongly depends on the size of oligomeric particles so that the smallest A β 1-42 oligomers with particle size of 1–2 nm (corresponding to dimers – pentamers [2]) were the strongest immunogens in comparison with 5–10 nm oligomers or broad size range oligomers. Moreover, the analysis of IgG subtypes in the sera of immunized mice revealed IgG2a as the major IgG subclass of A β -specific antibodies thus suggesting the predominant role of Th1 cells in B cell activation (Table 1). On the other hand, it cannot be excluded that IgG subtype distribution was influenced by the use of adjuvant since A β 1-42 oligomers were non-immunogenic in mice when applied without it. Previous studies on the neurotoxicity of different A β 1-42 oligomers in rat brain cell cultures revealed that the most neurotoxic A β 1-42 oligomers are of size 1–2 nm [2]. Thus, the smallest 1–2 nm A β 1-42 oligomers possess both high neurotoxicity and immunogenicity. The nature of the link between those two properties of the smallest 1–2 nm A β 1-42 oligomers remains still to be elucidated.

Our study demonstrated that the N-terminal part of the A β 1-42 oligomers was accessible to B cells in active immunization and induced the development of A β 1-42 specific antibodies. The obtained data on the surface-

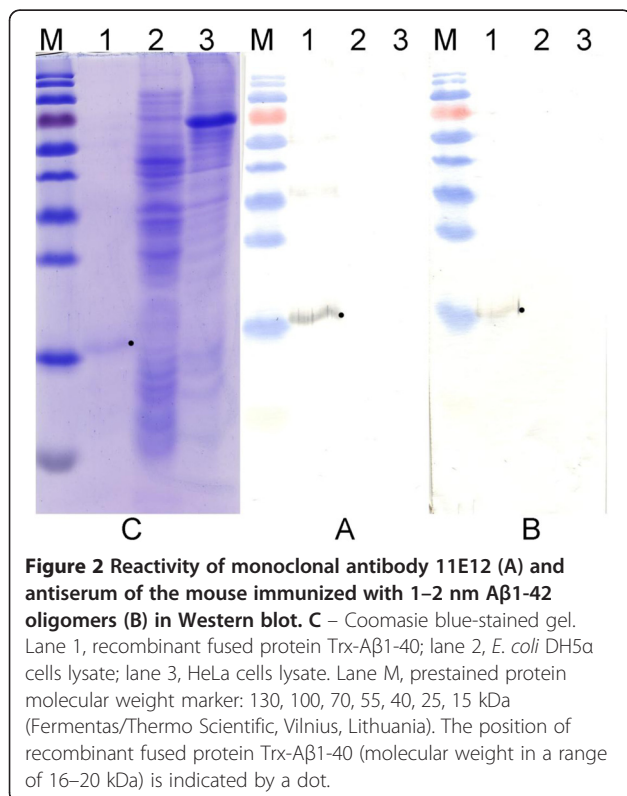


Table 2 Epitope mapping of A β 1-42 monoclonal antibody (MAB) 11E12 and polyclonal antisera based on their immunoreactivity with overlapping peptides spanning A β 1-42 sequence

Peptide No.	aa sequence	The reactivity of peptides with different antibodies in ELISA:			
		MAB 11E12	Antisera to broad size range oligomers	Antisera to 1–2 nm oligomers	Antisera to 5–10 nm oligomers
1 (aa 1–13)	DAEFRHDSGYEVH	++	+	++	+
2 (aa 7–19)	DSGYEVHHQKLVF	-	-	+	-
3 (aa 13–25)	HHQKLVFFAEDVG	-	-	-	-
4 (aa 19–31)	FFAEDVGSNKGAI	-	-	-	-
5 (aa 25–37)	GSNKGAIIGLMVG	-	-	-	-
6 (aa 31–42)	IIGLMVGGVIA	-	-	-	-

Every next peptide was starting with the same 7 aa from the previous one. The last peptide was 12 aa in length. The interaction between peptides and antibodies in an indirect ELISA is expressed as “++” – strong (OD > 1.0), “+” – weak (1.0 > OD > 0.25) and “-” – none (OD < 0.25).

exposure of the N-terminal part of A β 1-42 oligomers are in line with previous studies proposing that the assembly of oligomers starts at the middle and carboxy-terminal (C-terminal) parts of the A β [13,14]. Therefore, active immunization with A β oligomers may lead to generation of N-terminus specific antibodies. The immunogenicity of the N-terminal part of A β 1-42 oligomers also indicates that the oligomers tend to maintain their structural features during the interactions with B cells. This presumption is based on the fact that both monoclonal antibody 11E12 and polyclonal antisera raised against 1–2 nm A β 1-42 oligomers showed higher reactivity with A β 1-42 oligomers as compared to A β 1-42 monomers. On the other hand, both the antibody 11E12 and polyclonal antisera were reactive with synthetic peptides representing N-terminal linear epitopes of A β 1-42 thus suggesting that conformational epitopes were present but not immunodominant in A β 1-42 oligomers. Previous studies have demonstrated that passive immunization with a monoclonal antibody directed against the central domain of A β peptide (13–28 aa) was effective in reducing brain A β burden by promoting CNS and plasma A β clearance in mice [15,16]. Clinical trials on active immunization with A β 1-42 peptide (AN1792) revealed that plaque load has decreased, however this decrease was insufficient to improve the cognitive functions of AD patients [5]. It has been shown that the peptide AN1792 induced antibodies against the N-terminus of A β protein (1–8 aa) [17]. Thus, the A β oligomers used in our study resemble immunogenic properties of those explored in other immunization studies. In contrast, naturally occurring anti-A β antibodies have been shown to be mainly directed to the middle and/or C-terminal epitopes of A β protein and to inhibit the tendency of A β protein to fibrillate thereby blocking its neurotoxicity [18–20]. The latest studies have shown that these

naturally occurring antibodies bind to early oligomerization products and interfere with this process [11]. In contrast, antibodies raised against the N-terminus of A β protein may not necessarily interfere with the oligomerization of A β protein. These studies suggest that further assessments should be made for the development of new immunotherapy tools against AD using antibodies targeting either the middle or C-terminal part of A β oligomers.

In summary, our study provides new data on the immunogenicity of small-sized A β 1-42 oligomers and demonstrates their capability to induce antibodies directed predominantly to the N-terminal region of A β protein.

Conclusions

Small A β 1-42 oligomers of size 1–2 nm induce the strongest immune response in mice. The N-terminus of A β 1-42 oligomers represents an immunodominant region which indicates its surface localization and accessibility to the B cells. The results of the current study may be important for further development of A β -based vaccination and immunotherapy strategies.

Competing interests

The authors declare no competing interests.

Authors' contributions

ID was involved in the experimental design of the study, data collection, analysis and the manuscript drafting. RL generated the hybridoma. RB prepared and characterized the A β 1-42 oligomers, GV set up the assay for the preparation A β 1-42 oligomers, RM and VB contributed to the characterization of A β 1-42 oligomers, AZ conceived of the study, drafted and edited the manuscript. All authors read, approved and contributed to the final version of the manuscript.

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Author details

¹Institute of Biotechnology, Vilnius University, V. Graiciuno str. 8, LT-02241, Vilnius, Lithuania. ²Institute of Biochemistry, Vilnius University, Mokslininku str. 12, LT-08662, Vilnius, Lithuania. ³Institute of Neurosciences, Lithuanian University of Health Sciences, Eiveniu str. 4, LT-50009, Kaunas, Lithuania.

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